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Regulation of chitin synthesis in the larval midgut of *Manduca sexta*

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Abstract

In insects, chitin is not only synthesized by ectodermal cells that form chitinous cuticles, but also by endodermal cells of the midgut that secrete a chitinous peritrophic matrix. Using anti-chitin synthase (CHS) antibodies, we previously demonstrated that in the midgut of *Manduca sexta*, CHS is expressed by two cell types, tracheal cells forming a basal tracheal network and columnar cells forming the apical brush border [Zimoch and Merzendorfer, 2002, Cell Tissue Res. 308, 287–297]. Now, we show that two different genes, *MsCHS1* and *MsCHS2*, encode CHSs of midgut tracheae and columnar cells, respectively. To investigate MsCHS2 expression and activity in the course of the larval development, we monitored chitin synthesis, enzyme levels as well as mRNA amounts. All of the tested parameters were significantly reduced during molting and in the wandering stage when compared to the values obtained from intermolt feeding larvae. By contrast, *MsCHS1* appeared to be inversely regulated because its mRNA was detectable only during the molt at the time when tracheal growth occurs at the basal site of the midgut. To further examine midgut chitin synthesis, we measured enzyme activity in crude midgut extracts and different membrane fractions. When we analysed trypsin-mediated proteolytic activation, a phenomenon previously reported for insect and fungal systems, we recognized that midgut chitin synthesis was only activated in crude extracts, but not in the 12,000*g* membrane fraction. However, proteolytic activation by trypsin in the 12,000*g* membrane fraction could be reconstituted by re-adding a soluble fraction, indicating that limited proteolysis affects an unknown soluble factor, a process that in turn activates chitin synthesis.

Keywords: Chitin; Chitin synthase; Chitin synthesis; Midgut; Tobacco hornworm; Peritrophic matrix; Insect

1. Introduction

Chitin, a linear polymer of *N*-acetyl-*β*-D-glucosamine (GlcNAc), is the most abundant amino biopolymer in nature. It is used to support structural elements of various organisms because of its mechanical strength. In particular, it is found in arthropod exoskeletons and fungal cell walls, but also in other major Phyla such as annelids, molluscs and coelenterates (Wagner, 1994). Since chitin is not found in vertebrates and plants, enzymes involved in its synthesis constitute ideal targets for environmentally safe and selective agents used to control fungi-born diseases and insect pests. Chitin synthesis is catalysed by the enzyme chitin synthase (CHS) (EC 2.4.1.16), a large integral membrane protein that belongs to the family of *β*-glycosyltransferases. Most of the current knowledge on these important enzymes originates from investigations of CHSs from fungal sources. However, there are still many unsolved

Abbreviations: CHS, chitin synthase; DIG, digoxigenin; DTT, dithiothreitol; EDTA, ethylendiamine tetraacetate; GlcNAc, *N*-acetylglucosamine; HEPPS, *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Ms, *Manduca sexta*; PCR, polymerase chain reaction; PM, peritrophic matrix; RT-PCR, reverse transcriptase-polymerase chain reaction; UDP, uridine-5′-diphosphate

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questions to answer, mainly due to the lack of structural and functional data. In comparison to fungal systems, very little is known about insect CHSs, even though chitin synthesis is essential for growth and development. However, recent advances in the molecular biology of insect CHSs have taken a big step towards a better understanding of these important enzymes. During the last 4 years, CHS genes and cDNAs have been cloned from several species including dipteran insects like *Lucilia cuprina* (Tellam et al., 2000), *Aedes aegypti* (Ibrahim et al., 2000) and *Drosophila melanogaster* (Gagou et al., 2002), the lepidopteran *Manduca sexta* (Zhu et al., 2002) and the coleopteran *Tribolium castaneum* (Arakane et al., 2004). Insect CHSs appear to be encoded by only two genes, *CHS-A* and *CHS-B*, as deduced from *Drosophila*, *Anopheles* and *Aedes* genome resources as well as from genomic Southern blots (Hogenkamp et al., 2005; Arakane et al., 2004; Merzendorfer and Zimoch, 2003; Zimoch and Merzendorfer, 2002). Gene expression studies performed in *Lucilia*, *Tribolium* and *Manduca* indicated that *CHS-A* is specifically expressed in the epidermis and related ectodermal cells such as tracheal cells, while the expression of *CHS-B* has been suggested to be specific for gut epithelial cells that produce chitin in the context of peritrophic matrix (PM) formation (Arakane et al., 2004; Merzendorfer and Zimoch, 2003; Tellam et al., 2000). Most of the studies performed on chitin synthesis in insects so far have focused on the ectodermal isoform expressed by epidermal cells. Since the PM has pivotal functions for insects, we started to investigate chitin synthesis by midgut epithelial cells.

The PM is a secretion product that lines the intestine of most insects at one or more developmental stages. It facilitates digestion and protects the insect from invasion by microorganisms and parasites (Lehane, 1997). Insects have developed two types of peritrophic matrices. Type I peritrophic matrices are delaminated from the entire midgut epithelium, whereas specialized cells of the *cardia* produce type II peritrophic matrices. Insect peritrophic matrices usually exhibit a chitin content between 3% and 13% (Peters, 1992). However, a chitin content of even 40% of the dry weight has been reported for the type I PM of the tobacco hornworm (Kramer et al., 1995). This exceptionally high chitin content was one of the reasons why we started to investigate chitin synthesis in the midgut of this caterpillar. After cloning a cDNA encoding one isoform of the *Manduca* CHS, we could demonstrate CHS expression in the larval midgut of *Manduca* by Northern blots and reverse transcriptase-polymerase chain reaction (RT-PCR) (Zimoch and Merzendorfer, 2002). In situ hybridization, moreover, showed that columnar cells express the midgut CHS. The mRNA is localized at the apical region presumably co-localizing with the endoplasmic reticulum. CHS mRNA amounts were found to decrease from the anterior to the posterior midgut. Immunocytochemistry and immunoblots further revealed that CHS is enriched at the apical ends of microvilli forming the midgut brush border. Apart from midgut cells, we could detect CHS expression also in the epidermis and tracheal cells which both secrete a chitinous cuticle.

Chitin synthesis by midgut cells plays a key role in the intestinal biology of most insects and therefore may be a promising target for novel insecticides. In this paper, we report CHS expression studies and analysis of chitin synthesis in crude midgut extracts and membrane fractions. To analyse the relationship between biosynthesis and activity in the course of larval development, we examined mRNA and protein amounts as well as activity from 4th to 5th instar including molt and intermolt stages.

2. Materials and methods

2.1. Materials

Uridine-5′-diphosphate (UDP)-*N*-acetyl-D-[U-^{14}C]-glucosamine was purchased from Amersham and Ecolume scintillation fluid from ICN Radiochemicals. Nikkomycin Z and polyoxin D were obtained from Calbiochem and diflubenzuron from Riedel-de Haën. Most other chemicals were purchased from Sigma or Fluka.

2.2. Methods

2.2.1. Experimental animals

Larvae of *M. sexta* (Lepidoptera, Sphingidae) were reared under long-day conditions (16 h of light at 27 °C) using a synthetic diet modified according to Bell and Joachim (1974).

2.2.2. Tissue and cell extracts

All preparations were conducted at 4 °C. To obtain crude midgut extracts from *Manduca* larvae, the midguts of eight larvae were dissected and the gut content was completely removed including the PM to prevent fungal contaminations. Absence of fungal contaminations was verified by light microscopy. The tissue was washed three times with CHS reaction buffer (CHS buffer; 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 10 mM $MgCl_2$, 30 mM KCl, pH 6.5). Four midguts were homogenized in 3 ml CHS buffer for 60 s at 20,500 rpm using an ultraturrax T-25 device (Janke & Kunkel, Germany). For Western blotting 1/4 volume of a protease inhibitor mix (Rothistabi, Roth) was added before homogenization.

Membrane fractions were obtained by differential centrifugation as described previously (Cohen and Casida, 1980a). For that purpose, midgut tissues of

5th instar larvae (day 3) were homogenized with a glass/Teflon homogenizer in a 0.1 M sodium phosphate buffer (pH 7.4) containing 0.25 M sucrose and 0.15 M KCl. The homogenate was filtered through two layers of gauze and centrifuged at 12,000*g* for 30 min at 2 °C. The pellet was washed and resuspended in CHS buffer, whereas the 12,000*g* supernatant was filtered through silanized glass wool and centrifuged at 100,000*g* for 60 min at 2 °C. The supernatant was carefully removed and the microsomal pellet was washed and resuspended in CHS buffer.

2.2.3. Measurements of chitin synthesis

The assay for chitin synthesis was carried out according to a modified method of Archer (1977). The standard assay mixture was performed in CHS buffer containing 300 µg of protein in a total volume of 100 µl. The reaction was initiated by addition of 30 nCi UDP-*N*-acetyl-D-[U-^{14}C]-glucosamine (specific activity: 285 mCi/mmol, Amersham). Incubation was carried out for indicated time periods in 1.5-ml Eppendorf polypropylene tubes at 30 °C. The reaction was stopped by adding 1 ml trichloracetic acid (10%) or, in the case of alkaline digestion, by adding 1 ml 1.5 M KOH followed by an incubation for 2 h at 100 °C. Undissolved material was collected by filtration through 2.5 cm glass fibre filters (PALL; type A/E; 0.3 µm). The filters were subsequently washed with 10 ml of 98% ethanol, air-dried for 30 min at 50 °C and transferred to scintillation vials containing 5 ml Ecolume scintillation fluid (ICN). The radioactivity was measured as counts per minutes using a Beckman liquid scintillation spectrometer. Each experiment was repeated at least three times with each single value determined in triplicates.

For proteolytic activation of chitin synthesis, the reaction assays were incubated in the presence of 10 µg/ml trypsin (Sigma). The trypsin effect was neutralized by adding 10 µg/ml soybean trypsin inhibitor (Sigma).

2.2.4. RT-PCR analysis of *MsCHS1*/*MsCHS2* expression and alternate exon usage

Total RNA was isolated from dissected tracheal tissues from various stages of development using the RNeasy® Protect Mini Kit (Qiagen) according to the manufacturer's instructions. In each sample, two micrograms of total RNA were used as templates for cDNA synthesis using an oligo-(dT) primer. This cDNA then served as a template for the subsequent polymerase chain reactions (PCR). In order to minimize variations in primer annealing in the *MsCHS1*/*MsCHS2* expression analysis, a common forward primer was used that was designed from a region where the sequences of both transcripts are identical. The sequence of this primer was 5′-GAAAGGCGCTCATGGACG-3′, which spans position 2432–2449 in *MsCHS1* and 2398–2415 in *MsCHS2*. To allow for the simultaneous analysis of *MsCHS1*/*MsCHS2* expression, two reverse primers, specific for each transcript, were designed to produce different-sized PCR products when used in conjunction with the common forward primer. The sequences of these primers were 5′-TGAAGGAAGCCCAAGAGAG-3′ (spanning positions 3275–3293) for *MsCHS1*, and 5′-ACGTTGTTCAAATTGCATAGG-3′ (spanning positions 3120–3140) for *MsCHS2*. These primers were designed to have similar melting temperatures, enabling both PCR reactions to be carried out in the same vessel. The same strategy was used to examine the alternate exon usage of *MsCHS1*. Here, a common reverse primer was designed downstream of the alternate exon with the sequence 5′-TTCGTTATTAGCACCTAGGG-3′ (spanning positions 4466–4485). A forward primer specific for the *MsCHS1A* alternate exon with the sequence 5′-TGAAAGAATTGAGAGACTCG-3′ (located near the 5′ end of the alternate exon and spanning positions 3788–3807) and another forward primer specific for the *MsCHS1B* alternate exon with the sequence 5′-ATTACCTACATCGAGGAGAC-3′ (located near the 3′ end of the alternate exon and spanning positions 3919–3938) were used. For controls, primers for the constitutively expressed housekeeping gene encoding the ribosomal protein S3 (RpS3) were also used. All PCR reactions were conducted using the following conditions: denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s and polymerization at 72 °C for 1 min for 20 cycles.

2.2.5. Synthesis of hybridization probes and Northern blots

Purification of poly(A) RNA from different tissues of the tobacco hornworm was performed with the Quick-prep Micro mRNA purification Kit (Amersham) according to the manufacturer's instructions.

The PCR II TOPO vectors containing *MsCHS1* and *MsCHS2* fragments (nucleotide positions 4333–4588 and 4241–4477, respectively; see Hogenkamp et al., 2005) were cleaved with *Eco*R1 and the isolated inserts were ligated into pGEM-T$_{easy}$ (Promega). The resulting plasmids (pGEM-T–MsCHS1 and pGEM-T–MsCHS2, respectively) were linearized either with *Nco*I or *Nde*I, respectively, to obtain templates for in vitro transcription. Digoxigenin (DIG)-labeled RNA probes were generated by in vitro transcription using the DIG RNA-labeling Kit (Roche) and SP6 or T7 RNA polymerase producing either the sense or the antisense strand, respectively.

Northern blots were performed as described previously (Merzendorfer et al., 1997). To compare loading and to control RNA integrity after electrophoresis in 2% formaldehyde agarose gels, the RNA was stained with Radiant Red (BioRad) according to the manufacturer's protocol and documented with the Fluor-S Multi-Imager (BioRad). After transferring the RNA to

nylon membranes (Hybond N, Amersham), hybridization and stringency wash steps were carried out at 55 °C for *MsCHS*1 and at 60 °C for *MsCHS*2, respectively. Detection of radiolabeled RNA bands via anti-DIG antibodies (Fab fragments, Roche) conjugated with alkaline phosphatase was performed with CSPD (Roche) as a chemiluminescence substrate. Membranes were exposed to an X-ray film (Kodak X-omat AR) and band intensities were densitometrically quantified using the Fluor-S Multi-Imager and Quantity One software (BioRad). Intensities were measured in units of mean optical densities/mm^2.

2.2.6. Tracheal chitin staining

Anterior midgut tissues were prepared from 4th (4/5B) and 5th (5d1) instar larvae. After removing longitudinal muscles, the tissue was treated with paraformaldehyde-lysine-periodate fixative as described previously (Zimoch and Merzendorfer, 2002). After fixation, tissues were rinsed for 5 min at room temperature in phosphate-buffered saline (PBS; 20 mM KH_2PO_4, 20 mM NaH_2PO_4, 0.15 M NaCl, buffered to pH 7.3), incubated for 90 min in PBS containing 0.01% (w/v) Calcofluor white (Sigma), 0.1% Triton-X 100 and 2% BSA and washed three times for 30 min with PBS. The fluorescence of Calcofluor was visualized with an Olympus IX70 fluorescence microscope using a mercury short ARC photo optic lamp (HBO, Osram) and the NUA filter set (Olympus).

2.2.7. Other methods

Quantitative determination of GlcNAc by high-performance liquid chromatography was performed according to Anumula (1994). Protein determination using the Amido Black method, SDS-polyacrylamide gelelectrophoresis (SDS-PAGE), Western blotting and immunostaining were performed as described previously (Schweikl et al., 1989; Wieczorek et al., 1990; Zimoch and Merzendorfer, 2002). The band intensities of immunoblots were quantified densitometrically using the Fluor-S Multi-Imager and Quantity One software (BioRad).

3. Results

3.1. Two chitin synthase isoforms are expressed in the midgut

As shown elsewhere in this volume (Hogenkamp et al., 2005), the *Manduca* CHSs are encoded by two genes, *MsCHS1* and *MsCHS2*, with *MsCHS1* being alternatively spliced yielding *MsCHS1A* and *MsCHS1B* transcripts. *MsCHS1* is expressed in ectodermal cells with MsCHS1A prevalent in the epidermis. In contrast, *MsCHS2* was suggested to be expressed exclusively in endodermal midgut cells. The midgut of *Manduca* is a highly folded epithelium resting on a basal framework of muscles and tracheae, the latter of which provide highly metabolically active midgut cells with oxygen (Cioffi, 1979). Using gene-specific RNA probes for *MsCHS1* and *MsCHS2*, we looked for the expression of CHS isoforms in tracheae and midgut epithelium from 5th instar larvae. As shown in the Northern blot of Fig. 1, *MsCHS1* is exclusively expressed in tracheae, which have been carefully removed from the midgut to prevent contamination by other cells. By contrast, in the midgut epithelium (tracheae have been removed as much as possible) no *MsCHS1* transcripts were detectable in Northern blots. The opposite results were obtained for *MsCHS2* expression. While *MsCHS2* transcripts were found in the midgut epithelium, they were not detectable in tracheae. In agreement with the cDNA lengths for *MsCHS1* (5.2 kb) and *MsCHS2* (4.8 kb), the mRNA encoding *MsCHS2* migrated further in gel electrophoresis than that of *MsCHS1* (Fig. 1). Thus, *MsCHS1* is specifically expressed in tracheae lining the midgut, whereas *MsCHS2* is specifically expressed in midgut epithelial cells in the 5th instar. The results obtained are in good agreement with previous immunocytochemical studies performed with polyclonal anti-CHS antibodies, which showed that CHS is expressed in tracheal cells and apical microvilli

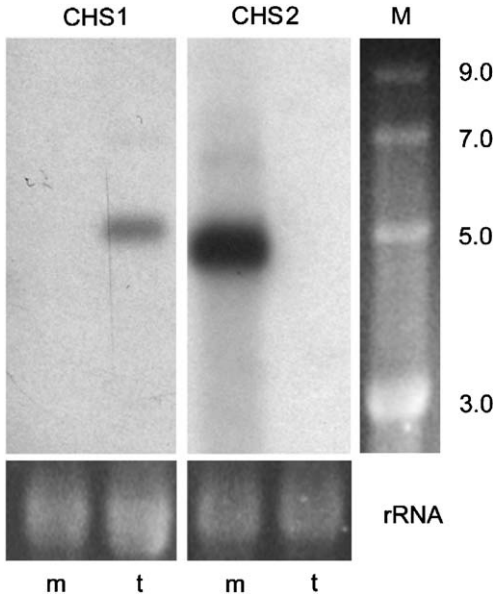

Fig. 1. *MsCHS1 is specifically expressed in tracheal cells and MsCHS2 in midgut cells.* Poly(A) RNA of 5th instar larvae was isolated either from abdominal tracheae (t) or from midguts (m) having removed residual tracheae as far as possible. After gel electrophoresis and Northern blotting, hybridization was performed with digoxigenin-labeled ssRNA probes complementary to either *MsCHS1* (CHS1) or *MsCHS2* (CHS2). To control loading and to determine fragment length ribosomal RNA (28S) and an RNA standard (M), respectively, were stained after gel electrophoresis with Radiant Red.

of midgut columnar cells (Zimoch and Merzendorfer, 2002).

3.2. Midgut chitin synthesis is regulated differentially during development

In order to compare expression of *MsCHS1* and *MsCHS2* in the course of larval development, we performed Northern blots tracing relative mRNA amounts for both isoforms from feeding 4th instar to the wandering stage of the 5th instar. As shown in Fig. 2, *MsCHS1*-mRNA levels drastically increase in poly(A) RNA from the midgut (including tracheae) at the beginning of the larval molt from 4th to 5th instar with a maximum at molting stage 4/5B (staging was performed according to Baldwin and Hakim, 1991). In the course of the 5th instar intermolt period, almost no *MsCHS1*-mRNAs were detected in Northern blots, as was also the case for the early wandering stage. Similar results were obtained by RT-PCR using an *MsCHS1* specific primer pair and cDNA obtained from isolated tracheae (Fig. 3). Only minor amounts of *MsCHS1*-mRNA were detected during the 5th intermolt period reaching a minimum at the second day of this stage, while at days 2 and 4 of the wandering stage (Wd2, Wd4; larval/pupal molt) *MsCHS1*-mRNA amounts significantly increased and declined again at the beginning of pupation (Pd0). The same trend was observed when primer pairs were used that discriminate between the two splice variants *MsCHS1A* and *MsCHS1B*. Interestingly, *MsCHS1B* appears to be preferentially expressed in tracheae, whereas *MsCHS1A* is preferentially expressed in the epidermis (Hogenkamp et al., 2005). The question remained as to why the tracheal CHS is expressed during the larval molt and not during the intermolt period. During each larval molt the total

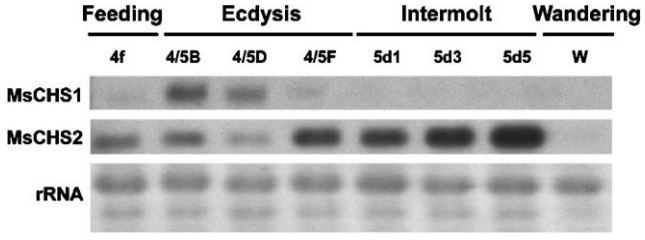

Fig. 2. *Northern blot analysis of MsCHS1 and MsCHS2* expression *during larval development*. Poly(A) RNA was prepared from larvae of different developmental stages between 4th and 5th instar (staging was performed according to Baldwin and Hakim, 1991). After the Poly(A) RNA was separated by gel electrophoresis, it was blotted onto nylon membranes. Hybridization was carried out with specific digoxigenin-labeled ssRNA probes complementary to *MsCHS1* and *MsCHS2*. In order to control gel loading, ribosomal RNA was stained with Radiant Red before blotting and visualized by UV illumination. f, feeding stage; molting stages: 4/5B, 4/5D, 4/5F; intermolt stages: 5d1, 5d3, 5d5 at day 1, 3 and 5, respectively; W, late wandering stage.

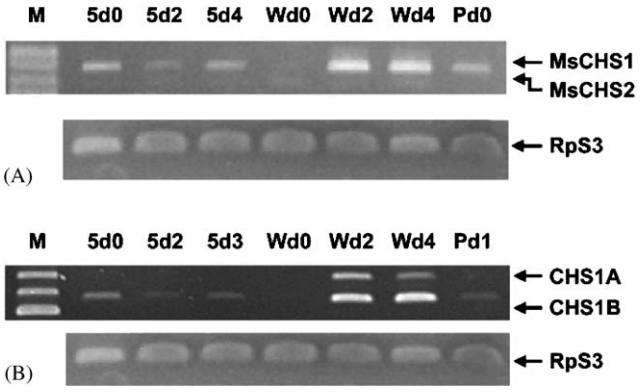

Fig. 3. *RT-PCR analysis of MsCHS1A/B and MsCHS2* expression *in tracheae during larval development*. RT-PCR using total tracheal RNA from different developmental stages was performed with primer pairs specifically detecting either *MsCHS1* and *MsCHS2* (A), or *MsCHS1A* and *MsCHS1B* (B), respectively. After agarose gel electrophoresis of PCR products and a DNA standard (M), fragments were stained with ethidium bromide and visualized under UV light. To validate the reactions with respect to developmental differences in RNA amounts, RT-PCR with a primer pair specific for the RNA of constitutively expressed ribosomal protein S3 (RpS3) was performed. Intermolt stages: 5d0, 5d2, 5d3 from days 0, 2 and 3, respectively; wandering stages: Wd0, Wd2, Wd4 from days 0, 2 and 4, respectively; pupal stage: Pd0 from day 0.

number of midgut columnar and goblet cells that differentiate from basal stem cells is quadruplicated (Baldwin and Hakim, 1991). As a consequence of epithelial growth during the molt, the need for oxygen supply increases. Therefore, tracheae and tracheoles have to grow coevally with the midgut epithelium during the molt. Tracheal growth can be visualized by treatment with Calcofluor white that effectively stains tracheal cuticles. As shown in Fig. 4, the tracheal network supporting the midgut epithelium exhibits more branching points and is more intensively stained by Calcofluor white during the 5th instar intermolt (day 1) than during the molt, indicating tracheal growth during the molt.

MsCHS2 expression in midgut columnar cells appears to be inversely regulated during larval development when compared with *MsCHS1* expression in the tracheae. While *MsCHS2*-mRNA was not detectable at the late wandering stage (Wd4) and only minor amounts of *MsCHS2*-mRNA were detectable during the molt from the 4th to 5th instar, the mRNA amounts significantly increased in the course of the 5th intermolt stage, reaching a maximum at day 5 of this stage (Fig. 2). Thus, *MsCHS2* is expressed during periods at which the larvae produce a chitinous PM surrounding the food bolus. However, in phases of starvation during the 4th–5th instar molt or during the prepupal wandering stage, *MsCHS2*-mRNA is down-regulated along with the cessation of PM formation.

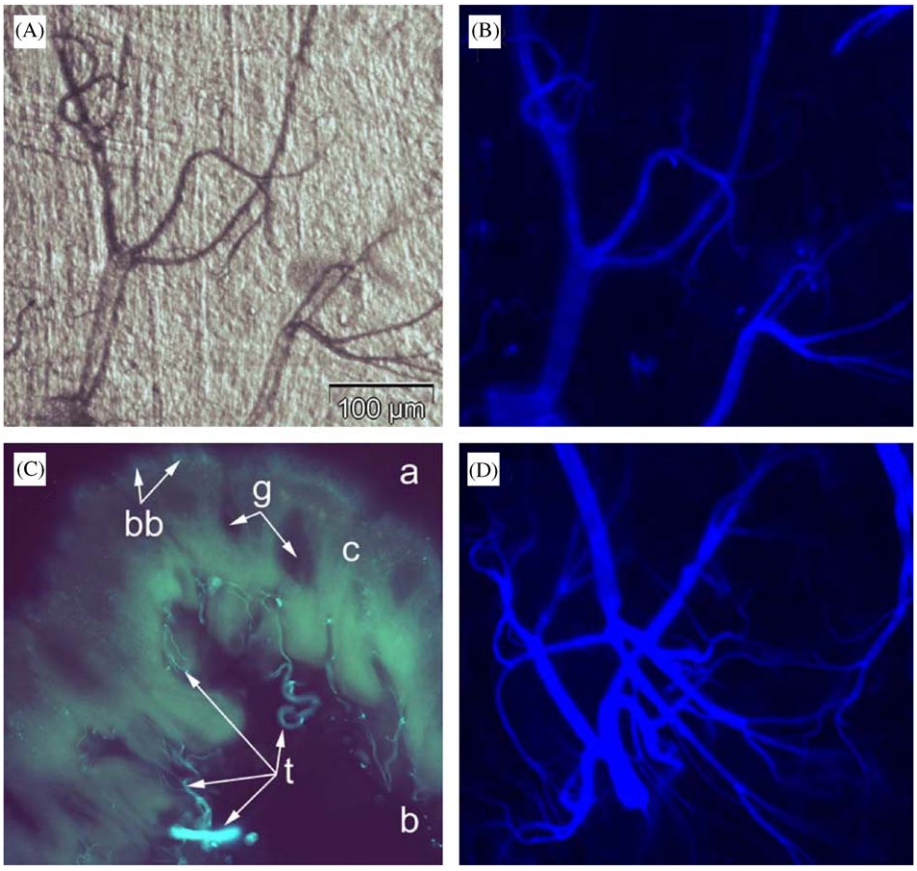

Fig. 4. *Morphology and growth of midgut tracheae during larval development of M. sexta*. The anterior midgut was prepared from 4th (molting stage 4/5B; A,B) and 5th (day 1; C,D) instar larvae. (A,B,D) After removing longitudinal muscles, the tissue was stretched and stained with Calcofluor white to visualize tracheae. Bright field (A) and fluorescence (B,D) was viewed with a Olympus IX70 microscope using the NUA filter set (typical images are depicted). (C) A 10 µm cryosection of the larval midgut was stained with Calcofluor and viewed with an Olympus IX70 microscope using an excitation wavelength of 480 nm and an NIBA filter set for emission. a, Apical; b, basal, bb, brush border; c, columnar cell; g, goblet cavity of a goblet cell; t, tracheae.

3.3. Chitin synthesis in crude midgut extracts

In order to investigate chitin synthesis by *Manduca* midgut cells, we performed activity assays based on the incorporation of the radiolabeled precursor, UDP-*N*-acetyl-D-[U-^{14}C]-glucosamine. As shown in Fig. 5A, crude midgut extracts of 5th instar larvae of *M. sexta* incorporate *N*-acetyl-D-[U-^{14}C]-glucosamine into an acid and ethanol-insoluble macromolecular fraction in a time-dependent manner. Since over 80% of the radiolabeled material was resistant to boiling potassium hydroxide, a versatile standard method to evaluate the portion of chitin in biological probes (Londershausen et al., 1988), we concluded that the macromolecular fraction consists mainly of chitin. This interpretation was also confirmed by measuring the increase of GlcNAc within the alkali-insoluble material after hydrolysing chitin with boiling HCl. We observed a net incorporation rate of about 0.4 nmol mg^{-1} h^{-1} GlcNAc with the crude extracts (data not shown). In control reactions without incubation, only low UDP-[U-^{14}C]-GlcNAc incorporations (1–4%) were obtained, indicating a low non-specific background. Addition of a large excess of non-radiolabeled UDP-GlcNAc displaced radiolabeled substrate, resulting in almost complete absence of radioactivity in the alkali-resistant pellet (Table 1). Incorporation was nearly linear with time (Fig. 5A), reaching a maximum at about 20 h, a value that is in the order of magnitude frequently found in arthropod systems (Spindler-Barth, 1997). It is possible that the observed incorporation of radioactivity is impaired by chitinase and protease activities present in the midgut extract.

To determine the optimum pH of the observed reaction, we measured chitin synthesis at varying pH values using different buffer components including 50 mM MOPS, HEPPS or MES, respectively (Fig. 5B). Highest incorporation was observed at pH 6–7. All succeeding experiments were therefore performed in MOPS buffer at pH 6.5 because of its non-chelating properties. To further evaluate chitin synthesis, we measured UDP-[U-^{14}C]-GlcNAc incorporation at

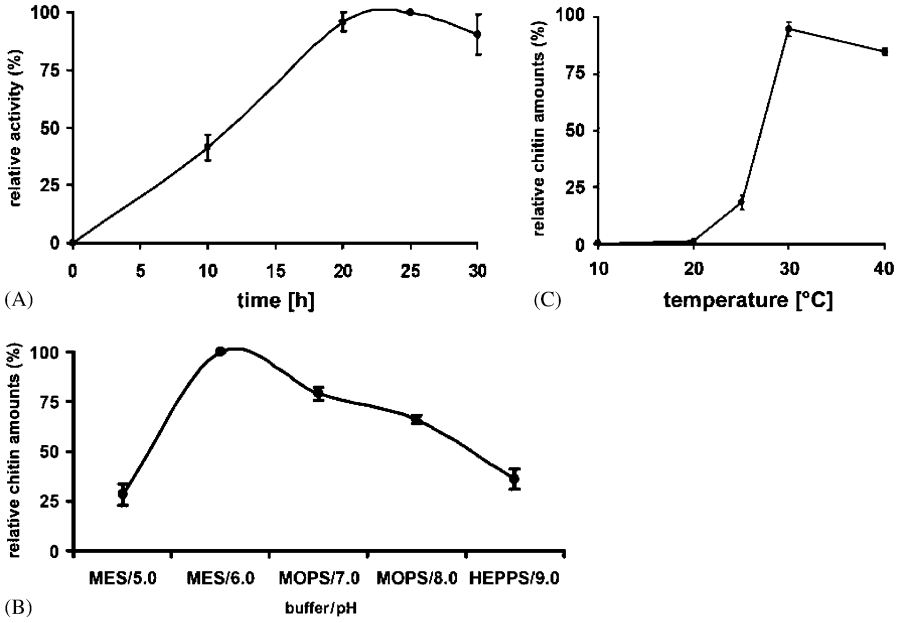

Fig. 5. *Chitin formation in the midgut of M. sexta*. (A) Relative chitin synthase activity was measured by the incorporation of *N*-acetyl-D-[U-^{14}C]-glucosamine into alkali-insoluble material obtained from crude extracts of the anterior midgut of 5th instar larvae (day 3–4). (B) The pH dependency of *M. sexta* midgut chitin synthesis. Relative chitin amounts of *M. sexta* midguts (5th instar, day 3) were measured after 16 h of incubation at the indicated pH in the presence of different buffers. Buffers used were 50 mM MOPS, 50 mM HEPPS and 50 mM MES. (C) Temperature dependency of *M. sexta* midgut chitin synthesis. Relative chitin amounts of *M. sexta* midguts (5th instar, day 3) were measured after 16 h of incubation at indicated temperatures. In each experiment, the amounts of radioactivity incorporated was measured with a scintillation counter and normalized to the maximal amount incorporated. Values are given in percent of maximal activity and as an average (±SEM) of three independent experiments.

various temperatures. Highest incorporation was observed at about 30 °C (Fig. 5C). Below 20 °C no traceable incorporation of UDP-[U-^{14}C]-GlcNAc was obtained, suggesting that the observed activity strictly depends on the membrane fluidity.

Chitin synthesis by the *Manduca* midgut exhibits similar characteristics to CHS activities previously reported for other insect and also fungal systems. Since divalent cations have been reported to stimulate CHS activity in other systems, we examined the influence of Mg^{2+}, Ca^{2+}, Mn^{2+} and Co^{2+} on activity (Cohen and Casida, 1980a, b; Machida and Saito, 1993). The addition of 10 mM Mg^{2+}, Ca^{2+} or Mn^{2+} (as chlorides) slightly inhibited incorporation, possibly by binding to negatively charged groups of the apical membranes. However, Co^{2+}, an ion previously shown in some systems to stimulate chitin synthesis, inhibited [U-^{14}C]-GlcNAc incorporation almost completely. The observed effects were not due to the anion, since similar results were obtained when we used nitrate and sulfate instead of chloride (data not shown). The presence of divalent cations in the reaction mixture is obviously indispensable also for midgut chitin synthesis, since EDTA moderately inhibited activity at a concentration of 10 mM and blocked it almost completely at a concentration of 50 mM. Interestingly, addition of sucrose and glycerol (the latter not shown) substances frequently added to stabilize proteins, also diminished incorporation of [U-^{14}C]-GlcNAc. GlcNAc, a molecule that is described as an allosteric activator of CHS activity (Merz et al., 1999), inhibited enzyme activity at comparably low concentrations of 1 mM as it was also reported previously for the stable fly (Mayer et al., 1980). ATP inhibited enzyme activity as well, but only at higher concentrations of about 10 mM, whereas UDP showed no significant effect at the same concentration. Di- and oligosaccharides such as *N*-*N*-diacetyl-chitobiose $(GlcNAc)_2$ or hexa-*N*-acetyl-chitohexaose $(GlcNAc)_6$ had clear inhibitory effects on midgut chitin synthesis at a concentration of 1 mM. An even stronger inhibition was observed, when a microsomal fraction was tested for the effects of these sugars. Addition of 1 mM $(GlcNAc)_2$ or 1 mM $(GlcNAc)_6$ led to an inhibition of [U-^{14}C]-GlcNAc incorporation by about 90%. In both cases, the hexasaccharide was slightly more efficient than the disaccharide. Reducing conditions established by the addition of dithiothreitol or the monothiol β-mercaptoethanol also clearly reduced incorporation rates. Addition of 40 mM DTT or β-mercaptoethanol (not shown) resulted in complete inhibition of the activity, similar to the situation previously reported for the *Tribolium* CHS (Cohen and Casida, 1980a, b). The classical CHS inhibitors nikkomycin Z and polyoxin D, well established inhibitors of fungal enzymes, exhibited only marginal effects. Diflubenzuron, an insect growth regulator in commercial

Table 1
Effect of various compounds on midgut chitin synthesis of *M. sexta*

Compound	Concentration	% Activity of control	
		Cell-free extract	Microsomal fraction
ATP	1 mM	101 (2)	
	10 mM	34 (4)	
UDP	10 mM	99 (4)	
Mg^{2+}	10 mM	88 (1)	
Ca^{2+}	10 mM	84 (6)	
Mn^{2+}	10 mM	85 (8)	
Co^{2+}	10 mM	2 (1)	
EDTA	10 mM	76 (1)	
	50 mM	2 (1)	
DTT	1 mM	69 (3)	
	20 mM	9 (4)	
	40 mM	0 (0)	
Sucrose	30% (w/v)	65 (5)	
GlcNAc	1 mM	38 (2)	
	10 mM	24 (4)	
UDP–GlcNAc (unlabeled)	10 mM	3 (1)	
Hexa-*N*-acetyl-chitohexaose	1 mM	25 (4)	10 (2)
N-*N*-diacetyl-chitobiose	1 mM	38 (1)	14 (5)
Trypsin	10 µg/ml	125 (2)	84 (4)
Trypsin + Trypsin inhibitor	10 µg/ml each	99 (6)	100 (5)
Trypsin inhibitor	10 µg/ml	102 (2)	104 (4)
Proteinase K	10 µg/ml	134 (3)	
Pefabloc SC	5 mM	73 (4)	76 (5)
Nikkomycin Z	20 µM	95 (5)	
Polyoxin D	20 µM	98 (6)	
Diflubenzuron	20 µM	100 (1)	

Relative chitin synthase activity from crude extracts of the midgut of 5th instar larvae (day 3–4) or from the microsomal 100,000*g* pellet was measured by the incorporation of *N*-acetyl-D-[U-^{14}C]-glucosamine for 16 h. Values are given in percentage of control and as an average (±SEM) of three independent experiments. Cations were added as chloride salts, but similar results were obtained with nitrate and sulfate salts.

use as an insecticide, had no effect on chitin synthesis by the *Manduca* midgut epithelial extracts (Table 1).

3.4. Proteolytic activation of midgut chitin synthase activity

In contrast to ectodermal CHSs, the midgut enzyme appears comparatively stable in crude extracts, since it exhibits moderate activity for more than 20 h at 30 °C. This may implicate a relative insensitivity of the enzyme to non-specific proteolytic degradation, possibly due to its constitutive exposure to high amounts of gut proteases. Interestingly, limited proteolysis stimulates chitin synthesis in various fungal and insect systems (Duran et al., 1975; Mayer et al., 1980; Cohen and Casida, 1980a, b; Hardy and Gooday, 1983; Machida and Saito, 1993; Merzendorfer and Zimoch, 2003). For yeast CHSs, it was postulated that at least some isoforms are zymogens whose proteolytic activation could trigger chitin synthesis at defined times and locations (Merz et al., 1999). To evaluate the influence of limited proteolysis, we measured the activity in the presence of porcine trypsin, an enzyme similar to one that is secreted by *Manduca* midgut epithelial cells into the gut lumen (Peterson et al., 1994). In the presence of trypsin, incorporation rates increased by about 25% as compared to control extracts without trypsin (Table 1). The stimulating effect of trypsin was eliminated by concomitant addition of soybean trypsin inhibitor, which itself had no effect on chitin synthesis. Similar effects were also obtained for a treatment with proteinase K, like trypsin a serine protease. The addition of 5 mM of the serine protease inhibitor Pefabloc Sc reduced incorporation of chitin precursor by about 25%. Taken together, these findings suggest that proteolytic activity may be important for stimulating midgut CHS activity.

Surprisingly, chitin synthesis is only activated by trypsin in crude extracts, but not in microsomal 100,000*g* fractions. By contrast, incorporation of [U-^{14}C]-GlcNAc was even slightly inhibited by the addition of trypsin to the microsomal fraction (Table 1). The effect of trypsin was abrogated by the parallel addition of soybean trypsin inhibitor that by itself again had no effect on chitin synthesis. The different effects of trypsin in crude extracts and microsomal fractions suggest that CHS activity is not directly affected by trypsin, but indirectly via the action of some unknown factor missing in the membrane fractions but present in the crude extract. In order to test this hypothesis, we tried to reconstitute trypsin-dependent proteolytic activation by separating and recombining different fractions that were obtained by centrifugation at 12,000*g* and 100,000*g*. Apart from the resuspended 100,000*g* pellet, chitin synthesis was detected also in the resuspended 12,000*g* pellet and again this activity was not stimulated by trypsin. Therefore, we recombined the 12,000*g* pellet with the resuspended 100,000*g* pellet as well with the 100,000*g* supernatant. Fig. 6 shows the result of the reconstitution experiment. While chitin synthesis in crude extracts is activated by trypsin (Fig. 6, lane 1), chitin synthesis in the 12,000*g* membrane fraction is unaffected (Fig. 6, lane 2) and in the microsomal 100,000*g* fraction (Fig. 6, lane 3) even slightly inhibited. As expected, the 100,000*g* supernatant did not exhibit significant activity (not shown). When the 12,000*g* pellet is combined with proportional amounts of the 100,000*g* pellet, trypsin activation cannot be restored. However, when proportional amounts of the 100,000*g* supernatant were added to the 12,000*g* pellet, we observed reconstitution of trypsin activation. Trypsin activation reached the same extent as observed for the crude

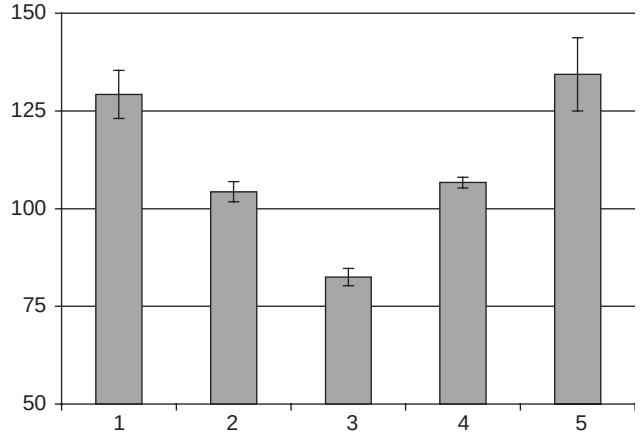

Fig. 6. *Proteolytic activation of chitin synthesis in different fractions of the larval midgut of M. sexta*. The midguts of 5th instar larvae were dissected and homogenized to obtain a crude extract. Successively, a 12,000*g* pellet, a 100,000*g* pellet and a 100,000*g* supernatant were obtained by centrifugation. The pellets were resuspended in proportional volumes of the assay buffer. To follow proteolytic activation, relative chitin synthesis was measured in the presence and absence of trypsin. (1) Crude extracts, (2) 12,000*g* membrane fraction, (3) 100,000*g* membrane fraction, (4) 12,000*g* membrane fraction after re-adding proportional amounts of the 100,000*g* membrane fraction and (5) 12,000*g* membrane fraction after re-adding the 100,000*g* supernatant. Values are given as percentage of activity in the absence of trypsin. Each measurement constitutes an average (±SEM) of three independent experiments.

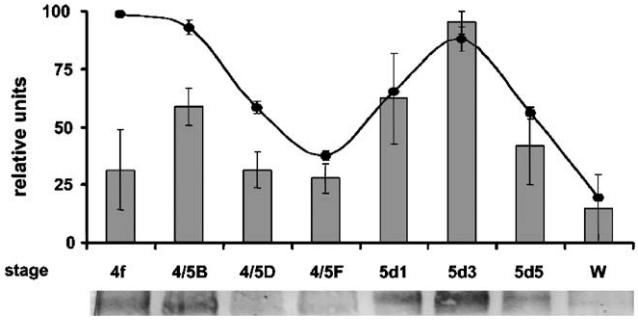

Fig. 7. *Changes in protein amounts and activity of the M. sexta midgut chitin synthase during larval development.* Crude extracts of the midgut were prepared from larvae at different developmental stages between 4th and 5th instar and protein amounts were determined (staging was performed according to Baldwin and Hakim, 1991). Each 5 µg was separated by SDS-PAGE, transferred onto nitrocellulose membranes and treated with a 1:10 dilution of anti-chitin synthase antibodies (Zimoch and Merzendorfer, 2002; lower figure). Immunosignals were quantified densitometrically using a FloroS Multi-Imager (vertical bars in diagram). Relative chitin synthase activities were determined as described elsewhere in this paper (curve in diagram). Values are given in percent of maximal activity and as an average (±SEM) of three independent experiments. f, Feeding stage; molting stages: 4/5B, 4/5D, 4/5F; intermolt stages: 5d1, 5d3, 5d5 at day 1, 3 and 5, respectively; W, late wandering stage.

extract. Thus, we conclude that chitin synthesis is not directly activated by trypsin-mediated proteolysis, but rather by proteolysis of an unknown soluble factor, a process that in turn activates chitin synthesis as a downstream event.

3.5. Chitin synthase activity and enzyme amounts during development

Midgut chitin synthesis is necessary for PM formation to envelop the gut content. Consequently, chitin synthesis occurs during periods of feeding, whereas during periods of starvation (such as molting) many insects interrupt PM production (Lehane, 1997). Thus, PM formation and along with midgut chitin synthesis vary depending on the insects' physiological conditions (Locke, 1991). To investigate regulation of chitin synthesis in the *Manduca* midgut, we looked simultaneously for activities and enzyme amounts in the course of larval development including the molting stages. Chitin synthesis was determined in crude extracts from larval midguts of different developmental stages. Enzyme amounts were estimated after SDS-PAGE by immunoblotting using polyclonal anti-CHS antibodies (Zimoch and Merzendorfer, 2002). Signal intensities were quantified densitometrically and compared with the respective measurements of chitin synthesis. As shown in Fig. 7, activities were found to be in good agreement with corresponding CHS amounts detected by immunoblotting. Both CHS activities and amounts decrease during molting and increase again after ecdysis when the larvae have started to feed again, thereby reaching a maximum on day 3 of the 5th instar. Moreover, in the prepupal wandering stage (day 4) only low activities and amounts could be detected (Fig. 7). The only discrepancy between activity and corresponding protein amounts was observed at the late 4th intermolt, when we observed high activities at moderate CHS amounts. Since chitin synthesis largely correlates with CHS amounts, we conclude that chitin synthesis is regulated mainly at the level of protein biosynthesis during larval development.

4. Discussion

RT-PCR and Northern blot analysis provided by Hogenkamp et al. (2005) and in this paper revealed that *Manduca* CHS isoforms *MsCHS1A/B* and *MsCHS2*, corresponding to the insect CHS gene classes *CHS-A* and *CHS-B*, respectively, are differentially expressed and regulated. While *MsCHS2* is expressed in columnar cells of the midgut, *MsCHS1A/B* is expressed in ectodermal cells of the epidermis and tracheal system. Interestingly, *MsCHS1A* transcripts are predominately found in epidermal cells and *MsCHS1B* transcripts in tracheal cells. So far, most studies performed on insect chitin synthesis have focused on chitin synthesis necessary for epidermal cuticle production. Since chitin

synthesis in the context of PM formation by midgut cells is essential for many insects, we decided to focus on the midgut CHS.

In the course of larval development, *MsCHS1* and *MsCHS2* expression is differentially regulated in the midgut. *MsCHS1*-mRNA levels are raised at the beginning of the molt, whereas *MsCHS2*-mRNA levels are clearly reduced at this stage. By contrast, *MsCHS1*-mRNA levels are reduced during the intermolt period, at which *MsCHS2*-mRNA levels are increased. The developmental pattern of expression of CHS isoforms meets the requirements for chitin synthesis during PM formation by the midgut and for cuticle formation by tracheal cells. PM formation occurs during the intermolt stage when the larvae feed and, correspondingly, *MsCHS2*-mRNA levels are high throughout this stage. Tracheal cuticle formation in the midgut occurs during the molt, since increasing numbers of midgut columnar and goblet cells have to be supplied with oxygen by newly formed tracheae. In Northern blots using midguts from molting stage 4/5B we observe a small increase of *MsCHS1* even though we tried to remove as many tracheae as possible. This finding may reflect intimate contact of growing tracheae to midgut epithelial cells. As visualized in Fig. 4C, tracheae migrate even in-between midgut cells, and therefore cannot be removed completely in our preparations. If tracheal growth primarily occurs in the more apical zones between midgut epithelial cells, the finding of *MsCHS1* expression in the midgut can be explained by the presence of growing tracheae in-between midgut cells.

Our conclusions regarding different physiological functions of CHS isoforms are in line with previous studies performed in insects. RT-PCR performed in *L. cuprina* revealed that *LcCHS1* transcripts are expressed in all life stages (Tellam et al., 2000). They were found primarily in the carcass (larva without internal tissues) and to a lesser extent in tracheae but not in the midgut or the *cardia*, suggesting that chitin synthesis for PM formation is catalysed by a different CHS, an assumption that was also supported by in situ hybridization. In *A. aegypti*, a cDNA encoding a CHS isoform corresponding to CHS2 was cloned and mRNA expression was analysed in midguts and whole females. *AaCHS2* (the revised correct denotation) was found to be expressed in the midgut in response to a blood meal (Ibrahim et al., 2000). In *Tribolium* significant *TcCHS1* transcript amounts were detected in embryos, last larvae, prepupae, pupae and adults, while *TcCHS2* transcripts were only found in last larvae and adults (Arakane et al., 2004). These findings have led to the suggestion that *TcCHS2* transcription is associated with chitin synthesis in the context of PM formation.

Measuring CHS activity in crude midgut extracts under various conditions revealed basic characteristics similar to that reported previously for other insect and fungal CHSs. As reported for other insect systems, chitin synthesis by the *Manduca* midgut exhibits an optimum pH at around pH 6–7, a temperature optimum at around 30 °C (Cohen and Casida, 1980a; Merzendorfer and Zimoch, 2003) and is inhibited by DTT (Cohen and Casida, 1980b) and glycerol at high concentrations (Mayer et al., 1980). It is strictly dependent on the presence of divalent cations, since addition of higher concentrations of EDTA blocks the reaction. In contrast to the CHS from the *Tribolium*, so far the only insect system in which chitin synthesis by midgut cells has been investigated (Cohen and Casida, 1980a, b), addition of Co^{2+} blocks activity almost completely, a finding that was also reported for the CHS of the stable fly, *Stomoxys calcitrans* (Mayer et al., 1980). The observed effect may be due to oxidation, because cobalt chloride is known to stimulate the generation of reactive oxygen species via a mitochondria-independent mechanism (Chandel et al., 1998). GlcNAc is discussed as an allosteric activator that regulates CHS activity (Merz et al., 1999). In *Manduca* midgut extracts, however, we observed a converse effect, since GlcNAc inhibited chitin synthesis effectively, as it was again reported in *Stomoxys* (Mayer et al., 1980). The simplest explanation for our observation may be that GlcNAc competes with the substrate at the sugar-binding site and that the proposed allosteric effector site is missing. The concept of GlcNAc acting in vivo as an allosteric regulator of CHS activity calls for experimental confirmation anyway due to additional conflicting observations. On the one hand, intracellular GlcNAc concentrations are known to be extremely low and, on the other hand, GlcNAc stimulation was reported to be completely lost upon isolation of the yeast enzyme (Kang et al., 1984). *N*-*N*-diacetyl-chitobiose and hexa-*N*-acetyl-chitohexaose inhibited chitin synthesis as efficiently as GlcNAc at a concentration of 1 mM, which may suggest that all these sugars bind to the same site. Binding studies with dimeric inhibitors recently provided evidence for the presence of two active sites in close proximity (Yeager and Finney, 2004), which could permit binding of at least the dimeric *N*-*N*-diacetyl-chitobiose.

To test different CHS inhibitors, we measured the effects of nikkomycin K, polyoxin D and diflubenzuron. All of them revealed only slightly inhibitory effects at best. Inhibition of midgut chitin synthesis, however, may be impaired by enzymatic metabolization. The insect midgut is a metabolically highly active tissue and known to be involved in efficient detoxification of insecticides (Smagghe and Tirry, 2001). Moreover, in the case of diflubenzuron, which is believed to act on an unknown pre- or postcatalytic step of chitin synthesis, it was reported that it does not inhibit chitin synthesis in cell-free preparations (Cohen, 2001).

In order to monitor chitin synthesis during larval development, we measured chitin synthesis in parallel with enzyme amounts. We found that both parameters decreased during 4th–5th instar molt and increased again after the larvae started feeding in the 5th instar. Having reached the wandering stage, chitin synthesis and enzyme amounts declined again. Parallel developing of activities and enzyme amounts suggests that chitin synthesis is regulated at the level of MsCHS2 biosynthesis. In line with this interpretation, a similar but not identical progression of *MsCHS2*-mRNA amounts at corresponding developmental stages was observed, indicating that developmental control of chitin synthesis occurs at least partly at the level of mRNA turnover. However, the finding that transcripts are detectable longer than enzymes at the late 5th instar (compare Figs. 2 and 7) may point to additional posttranscriptional regulatory mechanisms.

Beyond the regulatory mechanism discussed above, another level of control may be achieved in activating the enzyme during maturation. Limited proteolysis has been shown in numerous systems to activate CHS activity. Hence, it was suggested that the enzyme might exist as a zymogen (Cohen, 2001; Merz et al., 1999; Merzendorfer and Zimoch, 2003). In line with the zymogenic character of CHSs, trypsin treatment of *Manduca* midgut extracts led to an increase in chitin synthesis by about 25%, a magnitude that was found also in other insect systems, e.g. 29% in *Stomoxys* (Mayer et al., 1980) and 40% in *Tribolium* (Cohen and Casida, 1980a, b). The effect was eliminated by parallel addition of a trypsin inhibitor and could also be caused by proteinase K. However, when we tested a 12,000*g* membrane fraction, we observed a loss of proteolytic activation capacity that could be restored by adding the 100,000*g* supernatant. From these results, we concluded that proteolysis does not affect CHS directly, but a protease-sensitive soluble factor that is either an inhibitor of chitin synthesis or activated by proteolysis to stimulate chitin synthesis. The finding that the trypsin inhibitor did not affect CHS activity but the serine protease inhibitor Pefabloc may point to the involvement of another serine protease.

Cabib and Farkas (1971) already recognized that the zymogenic CHS from yeast could be activated either by trypsin treatment or by a soluble activating factor isolated from yeast cells. Moreover, early experimental indications were further substantiated suggesting that the zymogen and the activating factor reside in different cellular compartments (Cabib et al., 1973). Later on, Ulane and Cabib (1976) alleged that the activating factor is identical with proteinase B, a hypothesis that had to be revised after genetic experiments using proteinase B deficient yeast mutants (Silverman et al., 1991; Zubenko et al., 1979). After these experiments, the phenomenon of proteolytic activation became more and more puzzling and its significance is up to now not clear at all. In yeast, three different genes encode CHSs, *CHS1-3*. Chs1p and Chs2p, the corresponding gene products, are thought to be zymogens (Ford et al., 1996), while Chs3p was reported to exist in a non-zymogenic and a zymogenic state; the latter can be activated, as in the *Manduca* system, by different proteases. Its activity requires the expression of three further proteins, one of which, Cal2p, was suggested to function as an activator of the CHS Chs3p (Choi et al., 1994). *CAL-2* turned out to be allelic with the *CSD-4* and *SKT-5* genes and is frequently termed *CHS4* (Bulawa, 1993; Trilla et al., 1997; Sudoh et al., 1999). CHS4p is a 77 kDa protein, which is possibly attached to the membrane via prenylation at the *C*-terminus (DeMarini et al., 1997). CHS4p has been shown to stimulate zymogenic Chs3p activity by a protein–protein interaction and to function as an adaptor protein linking Chs3p to septins (DeMarini et al., 1997; Ono et al., 2000). Even though CHS4p has no known ortholog in insects so far, proteolytic activation is a common property of insect and fungal CHSs and may reflect a basic control mechanism. Therefore, it will be an exciting challenge to identify the soluble factor that we have postulated to affect chitin synthesis in response to proteolysis.

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